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Serum steroid concentrations and development of reproductive organs during puberty in male bonnethead sharks, *Sphyrna tiburo*

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Abstract

Puberty is a critical, hormone-mediated event during which an animal acquires the ability to breed and propagate. Despite the importance of this stage in animal reproduction, little is known regarding the physiological factors that regulate and/or accompany puberty in several vertebrate groups including elasmobranchs. To address the need for such information, the present study investigated morphological and hormonal changes that occur during puberty in male bonnethead sharks (*Sphyrna tiburo*). Serial changes in development of claspers, paired copulatory organs in male elasmobranchs, and serum steroid concentrations during puberty were evaluated in captive-held male *S. tiburo*. Captive-animal studies were supplemented by observations on gonadal development, gonaduct morphology, and serum steroid concentrations in feral, peripubertal male *S. tiburo*. Changes in size and histological architecture of testes and gonaducts of peripubertal sharks mirrored the seasonal progression of events that occur in these structures in mature males. Claspers grew in length continuously during puberty, but sharks did not reach functional maturity until a short period before mating activity commences in the mature population. Clasper growth appeared to be strictly regulated in *S. tiburo*, perhaps to ensure growth of these organs to sizes deemed critical for reproductive success. Serum concentrations of testosterone, dihydrotestosterone, progesterone, and 17β-estradiol increased in both captive and feral sharks during pubertal development, and may be associated with development of the gonads and gonaducts. Differences in hormone profiles of captive and feral sharks were observed at certain periods during puberty, but their origin remains unclear.

Introduction

Puberty is a critical stage in vertebrate reproduction during which a juvenile acquires the capacity to breed and propagate. This period is characterized by maturation of the gonads and accessory sex organs, which results from an activation of the endocrine system responsible for regulating reproductive processes, the brain-pituitary-gonad (BPG) axis. Since certain aspects of pubertal development (e.g., timing) dictate an animal's overall reproductive success, the factors that lead to and accompany this stage have been extensively studied, especially in birds and mammals (Paster 1991; Bourguignon and Plant 2000). In contrast, the regulation of puberty in lower vertebrates historically has received less attention. However, interest in this topic has steadily risen in recent decades, particularly due to increased use of sophisticated techniques for manipulating the timing of sexual maturation in cultured fish species. In addition, information on the factors that regulate puberty in lower vertebrates has become increasingly crucial for understanding the potential effects that environmental contaminants such as

endocrine disruptors can have on animal reproduction (Arukwe 2001; Jobling et al. 2002).

Despite rising interest concerning puberty in lower vertebrates, little is known about the processes that regulate and/or accompany this event in elasmobranchs (sharks, skates, and rays). Among other factors, the lack of information regarding this subject is likely due to the futility of developing commercial aquaculture operations for these fishes since they tend to exhibit life history traits consistent with low reproductive potential. However, since elasmobranchs are the oldest living animals that possess an archetypical vertebrate pattern of reproductive endocrinology (Callard and Klosterman 1988), information on the physiology of puberty in this group is likely to be enlightening from an evolutionary perspective. Furthermore, since recent studies indicate that certain elasmobranchs may be exposed to a variety of putative endocrine disrupting compounds during sexual maturation (Gelsleichter and Manire unpublished data), a greater understanding of the potentially sensitive factors that regulate this process is needed.

The objective of the present study was to investigate the physiological changes that accompany puberty in male bonnethead sharks (*Sphyrna tiburo*), an elasmobranch species with well-characterized reproductive traits (Parsons and Grier 1992; Parsons 1993; Manire et al. 1995; Manire and Rasmussen 1997). As documented by Parsons (1993), male *S. tiburo* are believed to reach full sexual maturity during their second year of life, based on dramatic growth and morphological development of external copulatory organs, the claspers. This report describes hormonal changes occurring in captive male *S. tiburo* maintained during the period of sexual maturation. Trends in serum steroid concentrations and the development of reproductive organs in feral, peripubertal male *S. tiburo* also were examined.

Materials and methods

Captive animal studies

Male *S. tiburo* used in captive animal studies were collected from sites within or adjacent to the Tampa Bay estuary in Florida using set gill nets. Pubertal status of male sharks was confirmed through examination of the claspers, which become elongated during this stage but are not yet calcified or capable of distal end flaring, properties of functionally mature individuals (Parsons 1993). Body lengths of all sharks used in this study were consistent with the size range of peripubertal male *S. tiburo* observed by Parsons (1993), a study conducted in the same geographical region. Following capture, sharks were transported to the laboratory in plastic bags containing seawater and oxygen. Sharks were maintained under natural conditions in a 12,000-l, outdoor circular tank capable of operating as an open or closed, recirculating system, and fed 5–6 times per week *ad libitum* with Atlantic thread herring or shrimp. After an acclimation period of approximately two weeks, all sharks were marked with nylon-barbed plastic dart tags for individual identification.

Observations on captive sharks took place during two consecutive years. In the first year of study, changes in body size, clasper size/morphology, and serum steroid concentrations were examined in four peripubertal male *S. tiburo* on a monthly basis during the 10-month period between February and November 2000. In the second year of study, the same factors were examined in four additional peripubertal male *S. tiburo* on a bi-weekly basis during the 6-month period between June and November 2001. The change in sampling interval in 2001 was intended to reveal variations in serum steroid concentrations that might have occurred over a semi-monthly time period and would therefore be undetected in the first year of study. During each sampling, measurements of total length and clasper size (defined as the distance between the anterior edge of the cloaca and the posterior tip of the clasper) were acquired for each individual. In addition, blood samples were obtained from each shark via caudal venipuncture and immediately placed on ice, where they were allowed to clot for 3–6 h. Blood samples were later centrifuged (1300 *g*) and serum frozen at 20 °C until thawed for measurement of four gonadal steroids believed to physiologically important in male elasmobranchs (Manire and Rasmussen 1997; Snelson et al. 1997; Heupel et al. 1999; Tricas et al. 2000): testosterone (T), dihydrotestosterone (DHT), 17β-estradiol (E_2), and progesterone (P_4). The acquisition of complete sexual maturity in captive sharks was signified by full calcification of the claspers, and confirmed by expression of semen from the urogenital papillae.

Feral animal studies

Sharks used to characterize trends in reproductive development and serum steroid concentrations in feral,

peripubertal male *S. tiburo* were collected from sites adjacent to Tampa Bay using set gill nets. Although these animals were obtained as part of a separate study that did not include the measurement of clasper size in standard sampling protocol, stage of maturity was determined based on clasper morphology. Only animals determined to be peripubertal using these criteria were used for the present study. Following capture, blood samples were obtained from each animal via caudal venipuncture and processed as previously described. Sharks were measured, weighed, and transported to the laboratory on ice for evaluation, measurement, and dissection of internal reproductive organs, which were used to illustrate changes in sexual maturation via histological examination. Vertebrae from the region below the first dorsal fin also were sampled from each animal for use in determining age at puberty.

For histology, samples of the testes, epididymides and seminal vesicles were fixed in 10% formalin (prepared in phosphate buffered saline modified for use with elasmobranch tissues, 10 mM NaH_2PO_4, 450 mM NaCl, pH 7.4) for 48 h, and then transferred to 70% ethanol for storage. Tissue samples were trimmed, dehydrated in a graded series of alcohols, cleared in a limonene-based solvent, and processed for routine paraffin histology. Tissue sections (5 μm) were prepared using a rotary microtome, adhered to poly-L-lysine-coated microscope slides, and stained with Harris hematoxylin and eosin for observations on tissue architecture using a compound microscope.

Age at puberty in male *S. tiburo* was determined using age estimates derived from counts of seasonally deposited rings in the vertebrae of feral, peripubertal sharks (Lombardi-Carlson et al., accepted). Individual vertebrae were sectioned longitudinally using a low-speed circular saw. Vertebral sections were adhered to microscope slides using toluene-based mounting media, and examined using a dissecting microscope and transmitted light. Using this procedure, a 'birth mark' formed during parturition and seasonally deposited annuli that are produced during winter months are visible as translucent bands that traverse the entire vertebral section (Parsons 1993). Since parturition in gravid female *S. tiburo* in the Tampa Bay region occurs in late summer (Parsons 1993; Manire et al. 1995), the first translucent band following the birth mark was considered to represent the first six months of growth. Therefore, the estimate of age for an individual shark was slightly less than the number of translucent bands present in its vertebrae. For example, a shark with one translucent band (i.e., the birth mark) was considered to be '0+ years old' whereas a shark with 2 translucent bands (i.e., the birth mark and the first winter annulus) was referred to as '0.5+ years old.' Vertebral sections were examined independently by two experienced readers to strengthen the accuracy of age estimates.

Hormone analysis

Concentrations of T, DHT, E_2, and P_4 in serum of captive-held and feral sharks were determined by radioimmunoassay (RIA) after purification by chromatography on Sephadex LH-20 microcolumns. Serum aliquots of 500 μl were extracted with 5 ml of freshly opened diethyl ether. The organic phase was decanted after freezing the aqueous phase in an ethanol/dry ice bath and the ether dried under a stream of air. Dried extracts were sequentially chromatographed on two different Sephadex LH-20 columns. On the first column (1.0 g LH-20 with an elution mixture containing hexane, benzene and methanol at 62:20:13 v/v), E_2 was separated from estrone and all neutral steroids. The neutral fraction from the first column was applied to the second column (2.5 g LH-20 with an elution mixture containing hexane, benzene and methanol at 85:15:5 v/v) and appropriate fractions for P_4, T, and DHT were collected. Purified steroids then were estimated using the RIA procedure fully described in Manire et al. (1995).

Data analysis

Temporal changes in serum steroid concentrations in captive-held sharks were analyzed for significance using one-way repeated-measures ANOVA (RM-ANOVA) with time of measurement as a within-subjects factor. The Huynh-Feldt correction factor (Huynh and Feldt 1976) was applied because data did not meet the assumption of sphericity (Mauchly's test of sphericity, $P < 0.05$). Pairwise comparisons between monthly and semi-monthly data sets were performed using Fisher's Least Significant Difference (LSD) test when significant variations were detected by means of RM-ANOVA.

Spermatogenic progression in the testis and development of the reproductive tract in feral, peripubertal male *S. tiburo* were assessed qualitatively using histological preparations. Sharks were grouped into distinct categories for stage of puberty on the basis of these observations, and trends in testis growth and serum steroid concentrations among and between specific categories were evaluated qualitatively. When specific

trends were apparent, data were grouped appropriately and compared using t-tests or one-way ANOVA followed by Student-Newman-Keuls method of pairwise comparisons to identify groups that differed significantly.

Results

Captive animal studies

In the first year of captive animal studies, average total length (TL) of peripubertal male *S. tiburo* gradually increased from 62.9 ± 1.01 cm to 76.0 ± 0.5 cm during the 10-month sampling period. Despite a 5-cm range in initial TL, body growth was markedly similar among all sharks (Figure 1a). In contrast, patterns of clasper growth were conspicuously different between certain individuals (Figure 1b). Sharks with an initial clasper length (CL) of approximately 5 cm experienced a rapid surge in clasper growth between June and September, whereas sharks with larger initial CL (~ 6.5 cm) experienced more gradual increases in the size of this organ. Despite these differences, CL was similar (mean CL $= 9.025 \pm 0.11$ cm) in all sharks by the time of full maturation, which occurred between late August and mid-September based on full development of claspers and presence of semen in the reproductive tract of each individual.

Serum concentrations of T, DHT, E_2, and P_4 increased significantly in captive-held sharks during pubertal development (RM-ANOVA, $P < 0.05$). Elevations in serum T concentrations occurred incrementally throughout the entire sampling period (Figure 1c), and were significant by the second month of study (Fisher's LSD, $P < 0.05$). By late summer, serum T concentrations in peripubertal male *S. tiburo* were similar with the maximum levels observed in feral, mature males, which occur during the same time period and appear to be associated with advanced stages of spermatogenesis (Manire and Rasmussen 1997). Thereafter, serum T concentrations in captive-held sharks continued to rise, a phenomenon not observed in mature males, which mate during the fall and experience a concurrent decline in serum androgen and P_4 levels (Manire and Rasmussen 1997). Unlike that observed for T, significant increases in serum concentrations of DHT, E_2, and P_4 did not occur until after mid- to late summer (Fisher's LSD, $P < 0.05$; Figure 1d–f). Following this initial rise, serum concentrations of these hormones continued to increase to levels comparable with those observed in mature males (Manire and Rasmussen 1997). However, as observed for T, reductions in serum DHT, E_2, and P_4 levels similar to those that occur in mature males during the fall mating season (Manire and Rasmussen 1997) were not observed in captive individuals. Associations between the rate of clasper growth and changes in serum steroid concentrations were not observed.

Due to the presence of one relatively small specimen, the range in initial TL of captive-held sharks examined in 2001 was approximately twice of that in the first year of study. Although this animal did not grow to the approximate size attained by other sharks (mean TL $= 76.33 \pm 0.67$ cm), rate of body growth was similar among all individuals (Figure 2a). As observed in the previous year, rate of clasper growth varied considerably in sharks with substantial differences in initial CL (Figure 2b). However, as in 2000, all sharks reached a similar CL (mean CL $= 8.5 \pm 0.09$ cm) by the time of maturation, which occurred between late August and mid-September.

Serum T concentrations in second-year animals increased significantly (RM-ANOVA, $P < 0.05$) from 88.5 ± 7.17 ng/ml to 247.1 ± 19.4 ng/ml between June and November (Figure 2c), a rise similar to that observed in first-year sharks during the same time period. In contrast, changes in serum DHT concentrations in these animals were not significant (RM-ANOVA, $P = 0.10$), but noteworthy differences among groups of individuals were observed (Figure 2d). Half of the sharks examined in 2001 experienced a conspicuous rise and subsequent decline in serum DHT levels in early fall, whereas concentrations of this hormone remained relatively unchanged in other specimens. Differences in serum E_2 profiles of sharks also were observed (Figure 2e), but consistent patterns among individuals were not apparent. Interestingly, a rise in serum E_2 concentrations occurred in all sharks at the end of the study period, and final concentrations of this hormone in some animals far exceeded normal circulating levels observed in feral males (Manire and Rasmussen 1997). Changes in serum P_4 concentrations in second-year sharks were marginally significant (RM-ANOVA, $P = 0.042$), and were similar to those observed in the first year of study (Figure 2f).

Feral animal studies

A total of 38 peripubertal male *S. tiburo* was collected from the Tampa Bay region between the months of March and November 1998. Sharks ranged from 60 to 75 cm in TL (Figure 3a), sizes consistent with

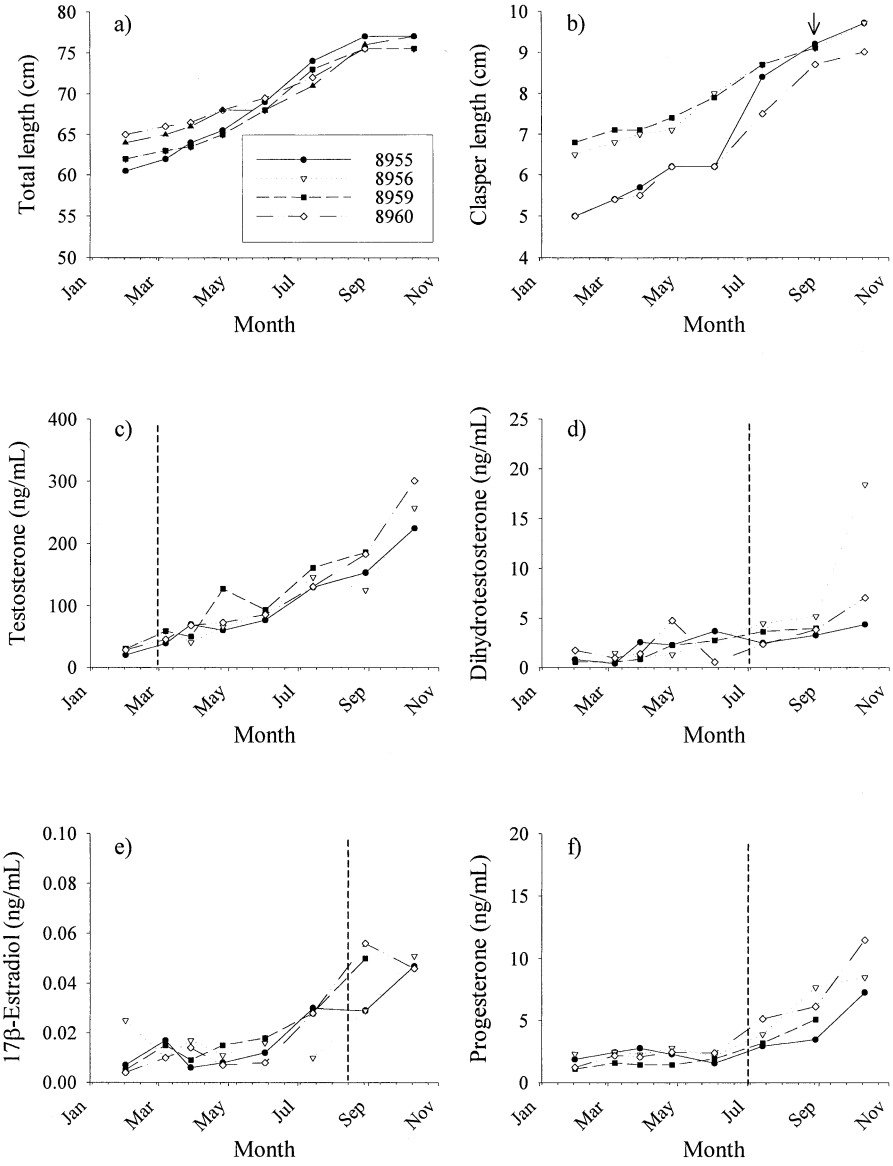

Figure 1. Temporal changes in total length (a), clasper length (b), and serum concentrations of testosterone (c), dihydrotestosterone (d), 17β-estradiol (e), and progesterone (f) in four captive male bonnethead sharks maintained during puberty in the first year of study. The 4-digit values included in the graph legend correspond to numbers printed on external tags used for animal identification. Arrows represents the period at which all sharks were deemed functionally mature based on presence of semen in the reproductive tract and full calcification of the claspers. Dotted lines represent the period after which serum steroid concentrations varied significantly ($P < 0.05$, Repeated Measures ANOVA followed by Fisher's Least Significant Difference test).

those previously observed for pubertal male *S. tiburo* by Parsons (1993). Age estimates were obtained for 35 of these sharks, whereas vertebral growth patterns from 3 individuals were deemed unreadable. Based on these estimates, the majority of pubertal sharks were in their first (Age 0.5+) or second (Age 1.5+) full year of life. However, male *S. tiburo* may undergo puberty as early as their year of birth (Age 0+) and as late as their third full year of life (Age 3.5+), based on the range in age-at-puberty of feral individuals (Figure 3b). This range was noticeably greater than that of size, but correlations between sexual development and size and age could not be tested and compared since all sharks were pubertal. A more complete description of growth rate in *S. tiburo* from this population is presented in Lombardi-Carlson (accepted).

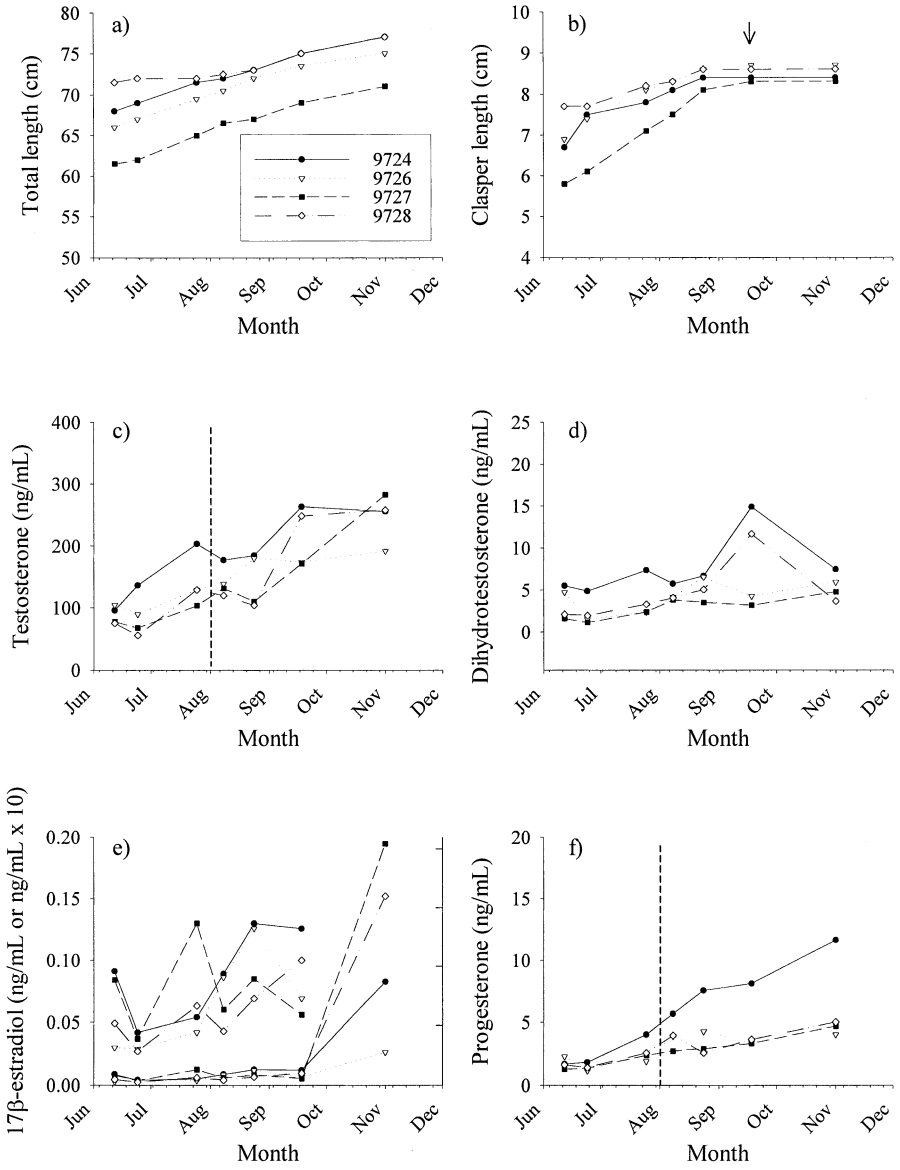

Figure 2. Temporal changes in total length (a), clasper length (b), and serum concentrations of testosterone (c), dihydrotestosterone (d), 17β-estradiol (e), and progesterone (f) in four captive male bonnethead sharks maintained during puberty in the second year of study. Serum 17β-estradiol concentrations are plotted twice using different units of measurement (ng/ml and ng/ml $\times$ 10) to illustrate the large increase in hormone concentrations during the end of the study period. The 4-digit values included in the graph legend correspond to numbers printed on external tags used for animal identification. Arrows represents the period at which all sharks were deemed functionally mature based on presence of semen in the reproductive tract and full calcification of the claspers. Dotted lines represent the period after which serum steroid concentrations varied significantly ($P < 0.05$, Repeated Measures ANOVA followed by Fisher's Least Significant Difference test).

Feral sharks were grouped into three categories for stage of puberty on the basis of histological observations. The first category (Stage I) included sharks that were in the earliest stages of gonadal development observed in this study, spermatogonial proliferation and meiosis (Figure 4a). Testes from these animals predominantly contained spermatocysts with secondary spermatogonia and primary/secondary spermatocytes, which have been classified as Stages 1–4 of spermatogenic progression in male *S. tiburo* by Parsons and Grier (1992). In addition to these gonadal features, epididymides and seminal vesicles in these individuals were generally small and poorly developed. The second classification (Stage II) comprised sharks that

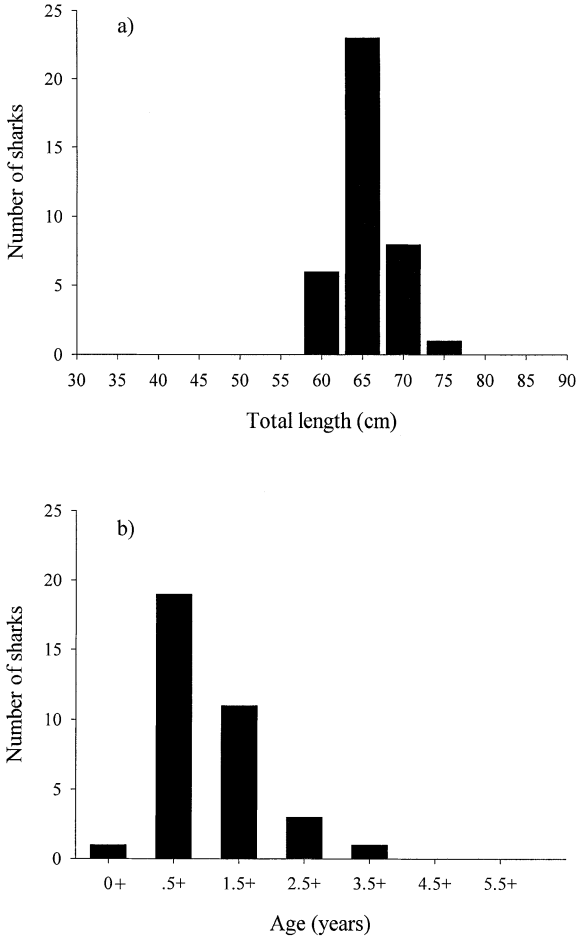

Figure 3. Size (a) and age (b) distribution of feral, peripubertal male bonnethead collected during the present study. Values for size refer to the total length of sharks in cm. Values for age refer to an estimate of chronological age, determined through analysis of vertebral annuli as explained in the text.

were undergoing gonadal spermiogenesis (Figure 4b); an event characterized by the presence of spermatids and/or loosely organized spermatozoa (Stages 5–6 in Parsons and Grier 1992) in testis preparations. Signs of advanced reproductive tract development were observed in these animals, including growth of the epididymal ducts and increased complexity of the seminal vesicle epithelium. Lastly, the final category of pubertal development (Stage III) included sharks that had acquired full gonadal maturity, a designation signified by the presence of tightly packaged, mature spermatozoa (Stage 7 in Parsons and Grier 1992) in the testis (Figure 4c). The reproductive ducts of Stage III individuals were large, well developed, and typically contained spermatozoa.

Temporal changes in the pubertal development of feral, male *S. tiburo* were apparent when sharks were grouped by stage of maturity and month of capture (Figure 5a). Between March and May, over 90% of pubertal males collected were categorized as Stage I or Stage II animals due to incomplete maturation of the testes and gonadal ducts. Full gonadal maturity of male *S. tiburo* appeared to take place by early summer based on increased occurrence of Stage III individuals during this period. In fact, spermatozoa were present in the epididymides of gonadally mature individuals as early as June. Testis size in Stage III sharks increased dramatically during late summer (August–September), then declined to previously observed dimensions in the fall (Figure 5b). Stage II animals were captured throughout the year, suggesting that acquisition of full gonadal maturity by the summer is not guaranteed for all individuals. Stage I sharks also were captured following summer, and presumably represent animals that will acquire gonadal maturity in the subsequent year. Contrary to that observed in Stage III individuals, an increase in testis size in Stage I and Stage II sharks did not occur during late summer.

Elevations in serum androgen and P_4 concentrations in Stage III sharks appeared to occur concurrently with the increase in testis size during late summer, based on visual examination of raw data (Figures 5c–d, f). When these data were grouped and statistically compared, only differences in serum T and DHT concentrations were found to be significant (Table 1). In contrast, changes in serum E_2 concentrations in gonadally mature individuals did not seem to occur in concert with variations in testis size (Figure 5e). Nonetheless, a significant decline in circulating E_2 concentrations in sharks of all stages took place between October and November (Table 1). Seasonal variations in serum steroid concentrations were not observed for Stage I and Stage II animals (Table 1). Furthermore, serum T and DHT concentrations in early-stage pubertal sharks were found to be significantly lower than those observed in Stage III animals during late summer (Table 1).

Discussion

Based on the results of the present study, male bonnethead sharks from the Tampa Bay region typically undergo puberty between the first and second full year following their birth. These findings are consistent with Parsons' observation (1993) that male

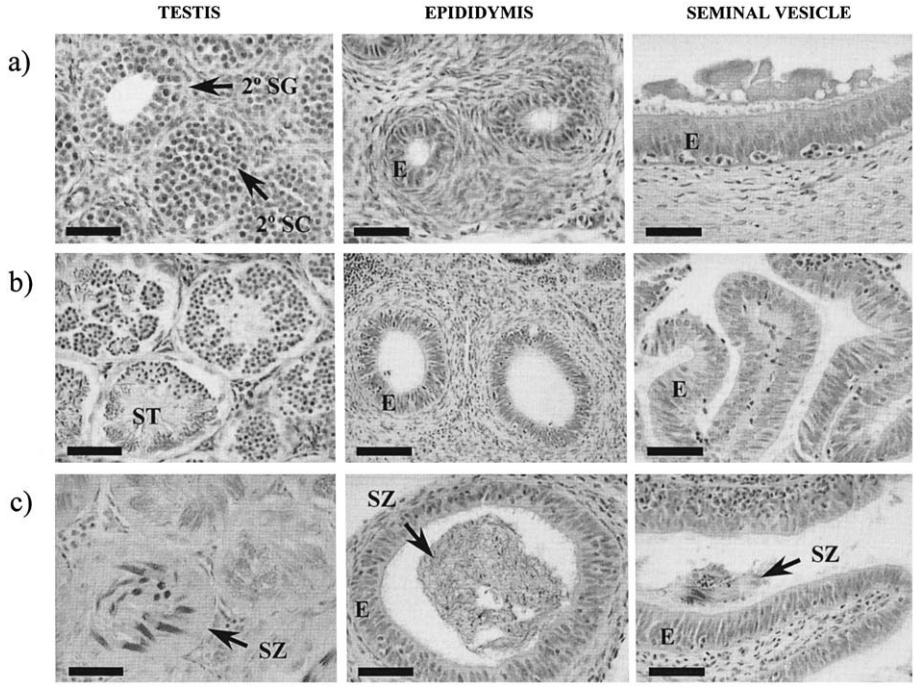

Figure 4. Histological architecture of the testis, epididymis, and seminal vesicle in feral Stage I (a), Stage II (b), and Stage III (c) pubertal male bonnethead sharks. 2° SG: secondary spermatogonia, 2° SC: secondary spermatocysts, ST: spermatids, SZ: spermatozoa, E: epithelium. Bar: 50 μm.

S. tiburo acquire functional maturity before they reach the end of their second year of life. However, timing of pubertal development in *S. tiburo* may be more associated with growth than chronological age, based on the greater range in age versus size of peripubertal individuals. Although limited in scope, these observations may suggest that the initiation of puberty in elasmobranchs is at least partially regulated by the environmental, metabolic, and/or hormonal factors that influence growth rate, such as food availability, nutrient absorption, and the growth hormone-insulin-like growth factor-I axis. Evidence for this premise has been observed in several other vertebrate groups, including bony fishes and mammals. For example, the precocious puberty that occurs in some salmonids has been linked with high rates of growth in these fishes (Le Bail 1988; Amano et al. 1997), and can be delayed by restricting food intake (Rowe and Thorpe 1990; Thorpe et al. 1990). Similarly, alterations of growth rate resulting from endocrine disorders, changes in nutrition, treatment with exogenous hormones, and/or other factors have been shown to influence the timing of sexual maturation in rodents and humans (Kennedy and Mitra 1963; Frisch et al. 1977; Bourguignon 1991). Future studies that directly examine the relationship between growth rate and pubertal development in elasmobranchs should be conducted to determine if this regulatory mechanism arose early in the course of vertebrate evolution (Huang et al. 1998).

As observed in several bony fishes (Cavaco et al. 1997; Holland et al. 2000; Amer et al. 2001), pubertal development of the testes in male *S. tiburo* appears to be similar to the normal progression of events that occur during sperm production in functionally mature individuals (Parsons and Grier 1992). Early stages of testicular development in feral, peripubertal males resembled the initial phases of gonadal recrudescence in mature sharks, a period characterized by nearly sole presence of pre- and peri-meiotic stage spermatocysts in the testes. The appearance of post-meiotic stage spermatocysts in testes of pubertal individuals occurred at approximately the same time period that spermatid production and spermiogenesis take place in mature males. Lastly, reductions in testes size in Stage III pubertal sharks occurred at the same time and to the same extent as that observed in mature sharks. Changes in the morphology of reproductive tract components in pubertal sharks also resembled those that occur seasonally in mature male *S. tiburo* (Gelsleichter et al. 2003). Seasonal changes in gen-

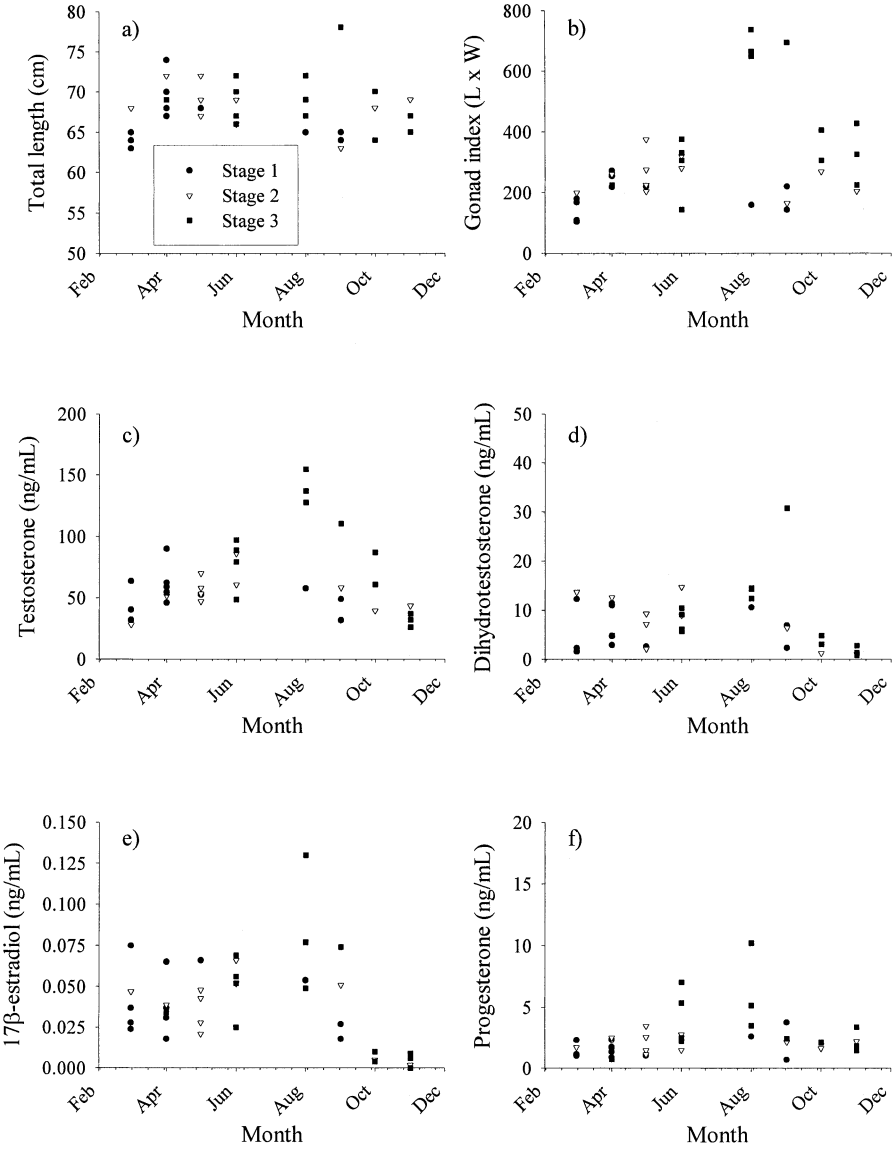

Figure 5. Total length (a), gonad index (b), and serum concentrations of testosterone (c), dihydrotestosterone (d), 17β-estradiol (e), and progesterone (f) in feral, male bonnethead sharks during different stage of pubertal development (Stages I, II, and III).

ital duct morphology also have been reported for other elasmobranch species, such as the small-spotted catshark, *Scyliorhinus canicula* (Garnier et al. 1999), and the Atlantic stingray, *Dasyatis sabina* (Piercy and Gelsleichter, unpublished data).

Rapid growth and structural modification of the claspers are common events in all male elasmobranchs during puberty, and are routinely used as diagnostic tools for determining stage of maturity in these fishes (Callard 1988). In the present study, the claspers of captive male sharks were fully developed by late summer, suggesting that many Stage III pubertal male *S. tiburo* acquire functional maturity by the fall mating season. In addition to these findings, this is the first study to demonstrate that the rate of clasper growth may vary in pubertal sharks, presumably in an effort to reach a size critical for reproductive success. Although little is known regarding the relationship between clasper size and successful reproduction in sharks, the length of this organ is likely to influence the ability of male sharks to efficiently deposit semen in sites within the female reproductive tract ideal

Table 1. Serum testosterone (T), dihydrotestosterone (DHT), progesterone (P_4), and 17β-estradiol (E_2) concentrations in feral, peripubertal sharks. Values are means (ng/ml) ± SE. Individuals were pooled following visual examination of raw data (see Figure 5) to permit statistical comparison between periods of sample collection and stages of puberty

Pubertal stage	Month	N	T	DHT	P_4	E_2
I & II	Mar – Apr	11	50.9 ± 5.4	7.8 ± 1.5	1.6 ± 0.2	0.04 ± 0.005
	May – Jun	7	61.1 ± 5.0	7.7 ± 1.6	2.0 ± 0.3	0.05 ± 0.007
	Aug – Sept	4	49.2 ± 6.2	6.5 ± 1.7	2.3 ± 0.6	0.04 ± 0.009
	Oct – Nov	2	41.5 ± 2.0	1.1 ± 0.1	1.9 ± 0.3	0.003 ± 0.001[a]
III	Mar – Apr	1	54.2	4.8	0.7	0.03
	May – Jun	4	78.5 ± 10.5	7.8 ± 1.1	4.3 ± 1.2	0.05 ± 0.009
	Aug – Sept	4	132.3 ± 9.2[a,b]	17.9 ± 4.3[a,b]	5.3 ± 1.7	0.08 ± 0.02
	Oct – Nov	5	48.6 ± 11.2	2.5 ± 0.7	2.2 ± 0.3	0.006 ± 0.002[a]

[a]significant difference in comparison with values obtained for the same stage during other sampling periods ($P < 0.05$, ANOVA followed by Student-Newman-Keuls method of pairwise comparisons). [b]significant difference in comparison with values obtained for Stage I & II animals during the same sampling period ($P < 0.05$, *t*-test).

for fertilization. As suggested for vertebrate intromittent organs in general, the evolution of clasper size in male elasmobranchs is almost certainly a consequence of competition between males for successful reproductive encounters (Birkhead 2000). The evolution of copulatory organs such as the clasper also is believed to be strongly associated with polyandry and the development of mechanisms that permit females to select between mates and/or their gametes (Eberhard 1985; Birkhead 2000).

Circulating levels of T increase during puberty in male *S. tiburo*, perhaps reflecting a role for this hormone in regulating development of the testis and/or accessory sex organs. The peak in serum T concentrations occurring during the final stages of gonadal maturation (i.e., late summer) is likely due to greater synthesis of this compound by post-meiotic spermatocysts, which has been demonstrated to occur in the testis of both *S. canicula* (Sourdaine et al. 1990; Sourdaine and Garnier, 1993) and the spiny dogfish, *Squalus acanthias* (Callard et al. 1985; Cuevas et al. 1993). Although T is largely produced by late-stage spermatocysts, its actions in the elasmobranch testis appear to be greatest in pre- and peri-meiotic cells based on the superlative number of androgen receptors expressed in these stages (Cuevas and Callard 1992). The effects of T and/or DHT on reproductive tract function in male elasmobranchs are more speculative due to a lack of published data regarding the distribution of steroid receptors in these tissues. While changes in serum androgen concentrations were not correlated with the rate of clasper growth in captive sharks, this is not entirely surprising because a direct role for androgens in clasper development has yet to be confirmed (Callard 1988).

While P_4 may serve as a precursor for androgen synthesis in male elasmobranchs, there are reasons to believe that it also may function in more direct regulation of the final stages of gonadal maturation in pubertal male *S. tiburo*. First, although serum P_4 and T concentrations in feral, pubertal sharks were their greatest during late summer, observations on captive sharks in 2000 indicate that patterns of change for these two hormones were not identical. Unlike serum T concentrations, which increased incrementally in captive sharks between early spring and late summer, circulating P_4 levels remained unchanged until the middle stages of spermatogenesis (i.e., mid-summer). These findings may reflect a role for P_4 in regulating spermiogenesis and/or spermiation; a hypothesis supported by the greater number of progesterone receptors in post-meiotic *versus* early-stage spermatocysts (Cuevas and Callard 1992). The lack of a correlation between T and P_4 concentrations in other male elasmobranchs, such as *S. canicula* (Garnier et al. 1999) and *D. sabina* (Snelson et al. 1997), also supports the notion that P_4 serves as more than merely a precursor for other steroids in the testis.

Serum E_2 profiles in feral pubertal sharks examined in the present study revealed little about the putative roles of this hormone in maturation of the testes and secondary sex organs. In contrast, data from captive sharks, particularly in the first year of study, suggested that circulating E_2 levels in puber-

tal male *S. tiburo* increase specifically during mid- to late spermatogenesis. These findings agree with Betka and Callard's observation (1998) that peri- and post-meiotic regions within the elasmobranch testis are largely responsible for E_2 synthesis, despite the fact that estrogen receptors are principally localized in pre-meiotic stage spermatocysts. Through production of E_2, which is transported to pre-meiotic spermatocysts via the intratesticular vascular system, late-stage spermatocysts are believed to regulate spermatogonial proliferation through a negative feedback system (Betka and Callard 1998). However, it should be noted that other studies on male *S. tiburo* have not acquired similar evidence for this process through examination of circulating E_2 concentrations (Manire and Rasmussen 1997).

The presence of steroid binding sites in the elasmobranch hypothalamus indicates that gonadal steroids are likely to influence the production and activity of gonadotropin-releasing hormone (GnRH) and, in turn, other components of the BPG axis (Jenkins et al. 1980). However, since no published studies have examined the effects of gonadal steroids on GnRH synthesis in elasmobranchs, the importance of hormonal feedback during puberty and other reproductive events cannot be clarified at the present time.

A conspicuous difference in serum steroid profiles of feral and captive sharks occurred in late fall, a period when circulating levels of all hormones remained elevated or continued to rise in captive individuals. In contrast, serum steroid concentrations in feral sharks declined during this period in a manner similar to that observed in mature male *S. tiburo* during and after the mating season (Manire and Rasmussen 1997). Although the reasons for these differences are unknown, it is intriguing to consider that they may be related to the lack of social interaction with female sharks in the captive environment, especially since exposure to potential mates can have significant effects on hormone production in many vertebrates (Silver 1993). Evidence for similar effects in elasmobranchs has been reported by Rasmussen et al. (1999), who observed differences in serum steroid concentrations of female clearnose skates (*Raja eglanteria*) that were housed with or without male conspecifics. As summarized by Silver (1993), such effects are largely mediated by chemosensory cues (i.e., pheromones), but may also result from tactile, visual, and auditory stimuli. Social communication between sexes also may occur via electrosensory cues in elasmobranchs, due to the presence of a highly sensitive and hormone-responsive electroreceptor system in these fishes (Tricas et al. 1995; Sisneros and Tricas 2000). Due to the reciprocal relationship between reproductive behavior and hormone levels in most vertebrates, further study is necessary to clarify the relationship between social context and endocrine status in sharks and their relatives.

The present study has provided the first glimpse of the hormonal *milieu* of male elasmobranchs during pubertal development, and an important basis for future studies on the regulation of puberty in these fishes. Although circulating levels of reproductive steroids undoubtedly mediate morphological and physiological changes that occur during puberty, it is important to stress that they are unlikely to serve as the primary impetus for sexual maturation. Therefore, future investigations on the regulation of puberty in sharks and their relatives should give attention to neuroendocrine secretions (i.e., gonadotropin-releasing hormone) in maturing animals, since they are generally considered to be the principal regulatory factors controlling the acquisition of reproductive maturity (Gore 2000).

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